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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

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Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

NON-HUMAN MAMMAL COMPRISING A MODIFIED SERCA2 GENE AND METHODS, CELLS, GENES, AND VECTORS THEREOF

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ENCLOSED APPLICATION PARTS (*check all that apply*)

[X] Specification *Number of Pages* 40
[X] Drawing(s) *Number of Sheets* 17
[X] Application Data Sheet. See 37 CFR 1.76
[ ] CD(s), Number
[ ] Other (specify):

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[ ] Applicant claims small entity status. See 37 CFR 1.27.
[X] A check or money order is enclosed to cover the filing fees.
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No [ ] Yes, the name of the U.S. Government agency and the Government contract number are: _____

[Page 1 of 1]

Respectfully submitted,

Date December 29, 2003

SIGNATURE Paul F. Fehlner

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Docket Number: 20057/0200598-US0

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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FEE TRANSMITTAL for FY 2004

Effective 10/01/2003, Patent fees are subject to annual revision.

☐ Applicant claims small entity status. See 37 CFR 1.27

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
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2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

		Extra Claims		Fee from below	=	Fee Paid
Total Claims	- ** =		x		=	
Independent Claims	- ** =		x		=	
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1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge – late filing fee or oath	
1052	50	2052	25	Surcharge – late provisional filing fee or cover sheet.	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	
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Application No.: TBA

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Specification, Claims & Abstract (57 pp) including (17) Dwgs Fig 1-8
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Application Data Sheet (2 pp)
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Application Data Sheet

Application Information

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NON-HUMAN MAMMAL COMPRISING A MODIFIED SERCA2 GENE AND METHODS, CELLS, GENES, AND VECTORS THEREOF

FIELD OF THE INVENTION

The present invention relates to a non-human mammal whose germ-cells and somatic cells comprise a modified sarco(endo)plasmic reticulum Ca^{2+} ATPase (Serca ATPase) gene. Further, the present invention relates to a method of inducing defective Ca^{2+} handling, as well as a method for studying heart failure in non-human mammals. The invention also relates to a modified Serca ATPase gene, as well as eukaryotic cells and vectors containing this gene. One purpose of the invention is to provide a tool to genetically affect Ca^{2+} handling in live animals in an organ of interest; e.g. in heart muscle to produce heart failure. The invention is particularly useful for studying heart failure and as a tool for developing novel drugs and methods for treatment of heart failure.

BACKGROUND OF THE INVENTION

Heart failure

Heart failure is defined as a pathophysiological state in which the heart is unable to pump blood to meet the body's metabolic demand. Heart failure has a high morbidity and mortality (2-5 years) after diagnosis. The number of heart failure patients has doubled the last 12 years in Western society, and results in a heavy load on the health-care system. The most common causes are high blood pressure, repeated episodes of ischemia, or myocardial infarction. Especially for the latter patient group, one expects an increase in heart failure at a later stage because of the increase in acute infarction survival rate.

Post-infarction heart failure.

After an infarction, the mechanical load on the remaining non-infarcted cardiac tissue increases. A hypertrophic process is induced in the cardiomyocytes (increase in cell mass and contractile proteins) and a restructuring of the extracellular matrix. These processes are often termed remodeling. With time, the heart is not able to compensate for the strain, and progressive failure develops. It is unclear whether the hypertrophic process itself is an integral part of failure progression, or whether hypertrophy/remodeling and failure are two parallel processes. In

general, the molecular mechanisms driving heart failure progression are not sufficiently understood, and the treatment regimens (β-blockers, angiotensin converting enzyme (ACE) inhibitors) are mainly directed towards alleviating symptoms – not the underlying mechanistic problems. Thus, new animal models that realistically mimic heart failure in humans, combined with a better understanding of the molecular mechanisms, may give new avenues for the development of therapeutic strategies.

Contractile function and molecular biology of heart failure

The three major determinants of contractile function in cardiac muscle cells are the trigger of Ca^{2+} release, reuptake of Ca^{2+} into the sarcoplasmic reticulum (SR) by Serca ATPases, and available SR Ca^{2+} content. Compared to normal cardiac tissue, the hallmark functional changes in post-infarction heart failure are an increase in the muscle relaxation time after contraction, reduced muscle fractional shortening, and reduced contractile force in the cardiomyocytes (Holt, Tønnessen et al. 1998; Hasenfuss and Pieske 2002). At the molecular level, it is well documented that the efficiency of pumping Ca^{2+} back into the SR via the Serca ATPases, in particular the Serca2 ATPase, is strongly reduced (Hasenfuss and Pieske 2002; Sande, Sjaastad et al. 2002; Frank, Bolck et al. 2003).

Serca ATPases are major ATP-driven Ca^{2+} handling ion pumps in the cell. "Ca^{2+} handling" refers herein to the transportation of Ca^{2+} inside the cell, in and out of organelles, and in and out of the cell. Serca proteins exist as several protein isoforms, coded by the three genes: Serca1, Serca2 and Serca3 (Loukianov, Ji et al. 1998; Wuytack, Raeymaekers et al. 2002, and refererences therein). Serca1 is predominant in fast-twitch skeletal muscle, Serca3 is expressed mainly in secretory tissues and in organs such as kidney and pancreas, whereas Serca2 is expressed in all tissues in the body. The Serca2a isoform is expressed in cardiomyocytes and slow-twitch skeletal muscle (very low levels in other tissues). In cardiac and slow-twitch skeletal muscle, Serca2 plays an essential role in muscle contraction. The Serca2b isoform is expressed in the vascular smooth muscle, CNS, and non-neuronal tissue. Recently, a new isoform expressed in monocytes, Serca2c, was described in humans (Gelebart, Martin et al. 2003). Muscle pathology has been associated with mutations in the Serca class genes in humans (Loukianov, Ji et al. 1998; Shull, Okunade et al. 2003). Deletion of Serca1 and Serca2 genes in mice is neoneatal and early

embryonic lethal, respectively (Periasamy, Reed et al. 1999; Pan, Zvaritch et al. 2003). In contrast, Serca3 null mice are viable, but display other defects (Arredouani, Guiot et al. 2002).

A number of studies have shown that Serca2 activity, Serca2 mRNA, and protein content in failing hearts of both humans and experimental animals are reduced compared with non-failing hearts. It has been clearly demonstrated that increasing Serca2 expression level, either by adenoviral infection or in transgenic mice, improved both contractility and SR function in experimental animals (Periasamy and Huke 2001; Nakayama, Otsu et al. 2003). It has also been demonstrated that Serca1 is able to partially substitute for Serca2 function in the heart (Loukianov, Ji et al. 1998).

The amino acid sequence similarity between the Serca1, 2 and 3 protein isoforms within one species is very high (>98%), as exemplified by the alignment of murine Serca protein isoforms shown in figure 1. Furthermore, all of the Serca proteins are highly conserved in mammals. This high level of conservation is exemplified by the amino acid alignment of Serca2 from a range of mammalian species provided in figure 2.

Serca and models of heart failure

The major disadvantages of traditional animal models where heart failure is induced by operative procedures are the difficulty of standardization, unintended post-operative effects on the animals, and the difficulty of large-scale experiments required for therapeutic drug development. Another major obstacle in studies on heart failure is the lack of methods to distinguish between primary causes of defective Ca^{2+}-handling and secondary adaptive processes. To date, no adult animal models with defective Ca^{2+}-handling to simulate heart failure have been developed, so drug screening and testing is rendered inefficient.

Given the role of Serca, particularly of Serca2, in heart failure and its high level of conservation throughout evolution, one approach to this problem is manipulation of the Serca2 gene in experimental animals. Two groups have previously developed mouse models with reduced Serca2 expression, however, none of these mice develop heart failure (Ji, Loukianov et al. 1999; Periasamy, Reed et al. 1999; Ver Heyen, Heymans et al. 2001; Antoons, Ver Heyen et al. 2003;

Shull, Okunade et al. 2003). While a homozygous null mutation in the Serca2 gene (*atp2a2*) in mice is embryonic lethal, $Serca2^{wt/null}$ heterozygous mice have a reduction in Serca2 protein and decreased contractility in the heart (Periasamy, Reed et al. 1999; Ji, Lalli et al. 2000; Huke, Liu et al. 2003). In a Serca2 isoform-specific mutant mouse model, $Serca^{2b/b}$ ($Serca2a^{null}$) animals develop cardiac hypertrophy (Ver Heyen, Heymans et al. 2001). Both models have a 30-40% reduction in Serca2 ATPase protein and activity. Differential changes in the expression of other proteins in these two models suggest different mechanisms of compensation for reduced Serca2 activity in these two models (Ji, Loukianov et al. 1999; Periasamy, Reed et al. 1999; Ver Heyen, Heymans et al. 2001; Antoons, Ver Heyen et al. 2003; Huke, Liu et al. 2003; Shull, Okunade et al. 2003). The fact that one allele of the Serca2 gene is inactivated at an early embryonic stage provides ample time to activate compensatory mechanisms. Hence, as heterozygous Serca2 mutants do not develop heart failure due to compensatory mechanisms from embryonic age, and homozygous Serca2 deletion mutants die in early embryonic life, the above animal models are inadequate for heart failure research and drug development.

The present invention overcomes the above obstacles by providing a transgene non-human animal, in which the level of Serca ATPase function, more particular Serca2 ATPase, can be directly manipulated in adult animals in order to simulate a defect Ca^{2+}-handling in general, and heart failure in particular. This effect is obtained by employing inducible recombination systems, for example Cre-loxP. Such systems allow both spatial (a given cell type) and temporal (a given time) control of mutations in living animals. The invention also comprises methods, cells, vectors and gene sequences thereof.

The system of Cre-loxP animals is based on two separate animal lines carrying two genetic components (described in U.S patent number 4,959,317). One animal line carries the gene of interest marked by loxP recombination sites (floxed gene). These sites are placed such that the gene of interest is inactivated when recombination takes place between the two recombination sites. This specific recombination reaction is catalyzed by the Cre recombinase. The second animal line expresses the Cre recombinase and directs the timing of gene destruction in the tissue or organ of interest. When these two animal lines are, for example, bred together, the gene of interest is excised in the tissue or organ of interest. A similar recombination system for mammals

has been developed, based on the frt recombination site and FLP recombinase for P1 bacteriophage.

The present invention is a valuable tool for studying medical conditions caused by a defect in Ca^{2+} handling, especially heart failure, as well as screening for, and further testing of, substances alleviating such conditions.

SUMMARY OF THE INVENTION

The present invention relates to a non-human mammal in which all cells contain genetic modifications in a Ca^{2+} handling Serca ATPase gene, more particularly the Serca2 ATPase. Defective Ca^{2+} handling is induced in live animals by introducing genetic elements which direct the timing and specificity of the gene inactivation to the organ or tissue of interest; e.g. cardiac muscle cells. The primary application is to provide a standardized and reproducible animal model for heart failure or other human diseases, in which the defective Ca^{2+} handling function by the Serca gene can be manipulated in live animals. This is the only existing animal to date with such genetic modifications for this class of Ca^{2+} handling proteins.

DESCRIPTION OF TABLES AND FIGURES

Table 1: Genotype distribution of breeding results for Serca2flox and MLC-*2v*-Cre knock-in mice.

Table 2: Body and organ weights for Serca2 cardiac heterozygous and control mice. Age, body, heart and lung weights of control and heterozygous animals (Mean ± SEM).

Table 3: Heart dimensions and flow parameters. FF, $Serca2^{flox/flox}$; FC, $Serca2^{wt/flox}$ MLC-$2v^{wt/cre}$; IVSd/s, inter-ventricular septum thickness in diastole respectively systole; LVDd/s, left ventricular diameter in diastole respectively systole; FS, fractional shortening in LVD; PWd/s, posterior wall thickness in diastole respectively systole; LAD, left atrial diameter; PWSV, posterior wall shortening velocity; PWRV, posterior wall relaxation velocity; LVOT, left ventricular outlet tract; RVOT, right ventricular outlet tract; CO, cardiac output; VTI, velocity time integral. All values are mean ± SEM

Table 4: Haemodynamic pressure measurements.

Table 5: Reduction of functional cardiac Ca^{2+} ATPase protein content in test model. Measurement of cardiac Ca^{2+} ATPase content in control and heterozygous animals (Mean ± SEM).

Fig. 1: Sequence alignment of mouse Serca 1, 2 and 3 protein. Amino acid identity is designated by a dot. Gaps are designated by -. Amino acid substitutions in the Serca family proteins relative to mouse Serca1 protein are shown. Accession numbers for mouse Serca ATPases: mouse 1a, NP_031530; mouse 2a and 2b, NP_033852 and J131870; Serca3a, AAB04099; Serca3b, AAB04098; Serca3c, CAA75746.

Fig. 2: Sequence similarity of Serca2 proteins in mammalian species. Amino acid identity is designated by a dot. Gaps are designated by -. Amino acid substitutions in the Serca2 proteins in other species relative to mouse Serca2 are shown. Accession numbers for Serca2 ATPases:

mouse 2a and 2b, NP_033852 and J131870; rat 2a, P11507; rat 2b, NP_058986; human 2a, NP_001672; human 2b, NP_733765; human 2c, AAO47398; dog 2a, O46674; cat 2a, Q00779; pig 2a and 2b, P11607; rabbit 2a and 2b, P20647.

Fig. 3: Targeting construct for Serca2 flox gene modification. Sequence information. Partial DNA sequence information of the targeting vector is given. Gaps in the sequence are given by lines and text of positioning in the *atp2a2* gene. Introns are in italics. Coding sequence is in capital letters. Extra inserted material (cloning sites, antibiotics cassettes and loxP sites) is underlined. LoxP sites are in bold. Elements are placed into atp2a2 gene intron 1, and intron 3 (2 positions). See figure 2 for schematic. The Serca2 gene sequence can be obtained from GenBank accession: NM_009722.

Fig. 4 A): Schematic representation of genetic manipulation. A simple representation of the genetic elements introduced into the Serca2 gene introduced into the animal invention. **B): Verification of Serca locus targeting events offspring from chimeric mice.** Offspring from chimeric animals were genotyped by PCR. The presence of flox recombination sites in introns 1 and 3 were verified by PCR using primers specific for intron 1 and specific for intron 3. DNA from Balb/C and from ES cell clone #1 were included as wild type and $Serca2^{wt/flox}$ allele controls. Fractions indicate dilutions of tail DNA preparations

Fig. 5: Specificity of gene deletion in a test model. Detection of Serca2 alleles in mouse tissues.

Fig. 6: Cardiac ANP mRNA expression. The expression level of ANP was estimated by Northern analysis. Blot were sequencially probed with [32P]-dCTP-labelled probe for ANP. Genotypes: (Control) FF = $Serca2^{flox/flox}$ (Cardiac heterozygous) FC = $Serca2^{wt/flox}$ MLC-$2v^{wt/cre}$

Fig. 7: Serca2 protein expression. Total protein homogenates of the indicated tissues from control and cardiac heterozygous Serca2 mice were run on 8% SDS-PAGE gels and analysed by Western blotting. Appropriate antibodies are given in the materials and methods section. Total lysate/lane: heart, 15µg, other tissues 40 µg. VV is heart lysate as positive control. Genotypes: (Control) FF = $Serca2^{flox/flox}$ (Cardiac heterozygous) FC = $Serca2^{wt/flox}$ MLC-$2v^{wt/cre.}$
A) S erca2a isoform in heart and soleus muscle
B) Serca2b isoform in heart, soleus and EDL muscle

Fig. 8: Compensatory mechanisms in Serca^{flox} MLC-2*v*-Cre mice. Total protein homogenates from hearts from control and cardiac heterozygous Serca2 mice were run on SDS-PAGE gels (7% for NCX1, 15% for phospolamban) and analysed by Western blotting. Gel loading was 10µg protein/sample except for detection of phosphoSer16 PLB (50µg/sample) and NCX1 in soleus + EDL (40 µg). Samples were denatured at 95 °C for 5-10 min (A, C) or at 37 °C for 15 min (B) to avoid complete disruption of PLB pentamers. Genotypes: (Control) FF = Serca2$^{flox/flox}$ (Cardiac heterozygous) FC = Serca2$^{wt/flox}$ MLC-2v$^{wt/cre}$

Panel A) Total phospholamban, phospho-Ser16 PLB, and phospho-Ser16 PLB, monomeric
Panel B) Total phospholamban, phospho-Ser16 PLB and phospho-Ser17 PLB, pentameric
Panel C) NCX1. The band present in EDL muscle is not NCX1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a non-human mammal all of whose cells comprise a modified Serca ATPase gene with inserted recombination sites. Said Serca ATPase gene modification may be homozygous or heterozygous; present as one or several copies, with or without the co-presence of a wild-type Serca ATPase gene. Furthermore, the Serca ATPase gene may be integrated in the genome of the host or exist in an extra-chromosomal vector. Any Serca ATPase type or isoform can be employed in view of the high level of sequence similarity and conservation of function of the Serca family (See figure 1), but preferably the ATPase gene is a Serca2 ATPase gene. Recombination sites can be inserted at any position adjacent or inside the Serca gene to cause complete or partial destruction of the gene when combined with a suitable recombinase. In general, any recombination sites suitable for eukaryotic cells can be used to carry out the present invention, like, for example, frt or loxP recombination sites. Preferably, the recombination sites are of heterogenous origin, more preferably of non-mammalian origin, and most preferably the recombination sites are loxP recombination sites. The recombination sites can be inserted in one or both alleles of the gene to cause a partial or complete destruction of Serca gene product activity. In a preferred embodiment of the present invention, the introduced loxP sites are placed such that both isoforms of the Serca2 genes (Serca2a and Serca2b) are disrupted in the tissue of interest when excision occurs. The shorter version Serca2a is expressed in the heart and in slow-twitch skeletal muscle, whereas the longer Serca2b protein is expressed in all tissues examined to date. Methods to introduce recombination sites into eukaryotic cells are known in the art.

The mammal of the current invention can further comprise a recombinase of non-mammalian origin. This recombinase must be able to interact with the recombination sites of the modified Serca ATPase gene and effect inactivation of the gene. When terms such as "destroyed", "disrupted", "destructed", "inactivated", or "excised" are used in relation to genes and the Cre-loxP system, or any similar system, it is herein meant to be construed as inactivation of a gene. The recombinase can be of any type (e.g. FLP or Cre recombinase) provided it does not affect

endogenous recombination sites; however, in a preferred embodiment the recombinase is a Cre recombinase. The recombinase gene can be expressed in all cells of the mammal or only in certain tissues like, for example, tissue-specific expression in skin, muscle, brain, kidney, or subgroups of these. In one specific embodiment of the present invention, a recombinase gene is expressed in the heart ventricle and in slow-twitch skeletal muscle. The recombinase gene can be exclusively, predominately, or modestly expressed in the tissue of interest compared to other tissues. In other words, the differential expression can vary greatly, but it is preferred that the recombinase is expressed exclusively or predominately in the tissue at stake. Furthermore, the recombinase gene can be expressed by induction, that is, controlled by a regulatory nucleic acid sequence, preferably an inducible promoter or regulatory nucleic acid sequence that controls the expression of the gene, or extra fused gene material which controls the intracellular Cre recombinase location in the cell. An endogenous or exogenous inducing agent can effect the activation of this inducible promoter or regulatory sequence. Examples of the former are the tet system promoter, while examples of the latter are fusion of mutant estrogen receptor domain to the recombinase which confers tamoxifen inducibility. Methods to introduce a recombinase gene and its promoter or regulatory sequence into a eukaryotic cell are within the knowledge of a person skilled in the art.

In view of the very high level of function and sequence conservation of the Serca ATPases amongst mammals, as shown in figure 2, the non-human mammal may be any mammal except a human being. Preferably the mammal is a non-human mammal generally used as animal models in pharmaceutical and scientific research and development, e.g. porcine, canine, or rodent animal models, more preferably a rodent, and most preferably a mouse. Methods and procedures for handling of experimental animals, experimentally and ethically, are within the scope of the art.

Another aspect of the present invention is a eukaryotic cell comprising a modified Serca ATPase gene with inserted recombination sites. The Serca ATPase gene, recombination sites, and regulatory nucleic acid sequences are as described *supra*. The cell is preferably of mammalian origin, more preferably of non-human mammalian origin, even more preferably of rodent origin, and most preferably of mouse origin. The cell may be of any particular type, for example, a

cardiomyocyte, a keratinocyte, a neuron, an embryonic cell, etc. Most preferably, the cell is an embryonic cell. The cell may further comprise a non-native or heterogenous recombinase gene, more preferably a non-mammalian recombinase gene, such as a FLP or Cre recombinase gene. Preferably, the recombinase gene is a Cre recombinase gene. Methods for transfecting or transforming cells, cloning genes and genetically modifying genes are well known within the art.

Yet another aspect of the present invention is a modified Serca ATPase gene with inserted recombination sites. The Serca ATPase gene, recombination sites, and regulatory nucleic acid sequences are as described *supra*. Suitable recombination sites can be inserted at any position adjacent or inside the Serca gene to cause complete or partial inactivation of the gene when present in a host cell. Any types of recombination sites can be used provided endogenous recombinases do not bind and effect recombination at the site when present in a host cell. Preferably, the recombination sites are of heterogenous origin, more preferably of non-mammalian origin. According to a most preferred embodiment, the recombination sites are loxP recombination sites. The ATPase gene, which is to be modified, is preferably a Serca2 ATPase, and most preferably as listed in Genbank SEQ ID NM_009722. According to a most preferred embodiment, said Serca ATPase is modified substantially as listed in SEQ ID 1 (Figure 3). Here, a modification of the flanks of the Serca2 gene, as employed in a most preferred embodiment, is depicted. The gene sequence of the Serca gene can be degenerated as a result of the redundancy of the genetic code. Also, codons can be changed with codons encoding similar amino acids. Methods necessary to enable the practice of the present invention are known within the art.

Another aspect of the present invention is a vector comprising the above Serca ATPase gene. The vector may be of any type appropriate for the task, for example, a shuttle vector, vectors for replication in eukaryotes or prokaryotes only, yeast vectors, etc. In a preferred embodiment the vector is based on pBluescript 2 KS.

The present invention also provides a (1) method for inducing defective Ca^{2+} handling in a non-human mammal and a (2) method for inducing heart failure in a non-human mammal. (1) The

first method comprises the steps of inducing recombination and inactivation of a Serca ATPase gene in a non-human mammal as described *supra*. This may be achieved by introducing a recombinase gene as described above into a non-human mammal, all of whose cells comprise a modified Serca ATPase gene with inserted recombination sites; and further induce expression of the recombinase gene to effect Serca inactivation. Introduction of the gene can be done, for example, at the embryonic level by embryo fusion or by gene-therapeutic methods like exposing a live animal to a modified adenovirus carrying a suitable recombinase. In the latter case, the virus can then be targeted to specific tissues of the animal. Traditional mating is, however, a preferred method for producing animals whose cells, all or in selected tissues, comprise the above described modified Serca gene and an inducible recombinase with affinity for the recombination sites employed. The gene introduction step can of course be omitted in cases where suitable model animals already have been produced. The non-human mammal animals are, as earlier described, preferably standard laboratory animals, like e.g. monkeys, pigs, dogs, sheep, goat, guinea pigs, or rodents, more preferably rodents, and most preferably mice. The Serca ATPase gene is preferably of type 2 and the recombination sites can be inserted at any position adjacent or inside the Serca gene to cause complete or partial destruction of the gene in combination with a suitable recombinase. Preferably, the recombination sites are of heterogenous origin, more preferably of non-mammalian origin. Most preferably, the recombination sites are loxP recombination sites. The recombination sites can be inserted in one or both alleles of the gene to bring about partial or complete destruction of Serca gene product activity. Methods to introduce recombination sites into eukaryotic cells are known in the art. The recombinase gene is preferably of heterogenous origin, more preferably of non-mammalian origin, and most preferred a Cre recombinase. The recombinase is preferably controlled by an inducible promoter sequence or another inducible regulatory sequence. It is preferred that the recombination gene is only, or predominantly, expressed in specific tissues, as described above. This can be any tissue, but most preferably heart tissue. (2) The second method comprises the steps of inducing recombination and inactivation of a Serca ATPase gene in heart tissue. This may be achieved by mating a non-human mammal with the above described modified Serca ATPase gene with a non-human mammalian partner, whose cells comprise a suitable recombinase gene expressed in heart tissue as described above; and further stimulate expression of the recombinase gene to induce Serca inactivation. However, any type of method can be used to produce animals with heart tissue

comprising both a modified Serca gene and a nonnative or heterogenous recombinase. These methods include for example gene therapy, embryo fusion, etc. The step of mating or gene introduction can of course be omitted in cases where suitable model animals already have been produced. The non-human mammal is preferably a standard laboratory animal, like exemplified in the method above. The recombinase gene is preferably controlled by an inducible promoter sequence or another inducible regulatory sequence, for example a tamoxifen-inducible promoter as described *supra*. The induction of the promoter can occur at any time; however in a specific embodiment induction occurs post-weaning, in another it occurs in a reproductively mature mammal, and yet another embodiment is on a mammal displaying all adult characteristics of the species. Depending on the modification of the particular Serca ATPase; for example, whether the modification is heterozygous or homozygous, or whether the destruction is partial or complete; Ca^{2+} handling in heart tissue is partially or fully disturbed by the tissue specific induction of the recombinase. The reduced Ca^{2+} pumping ability, depending on the level of reduction, will lead to effects varying from decreased heart contractility to massive heart failure. In a preferred embodiment of the present invention a rodent, all of whose cells comprise a Serca2 gene modified with loxP sites, is mated with a rodent partner, all of whose cells comprise a Cre recombinase, to produce progeny with cells comprising both said Serca2 gene and Cre recombinase gene. In a most preferred embodiment of the present invention a mouse; all of whose cells comprise a Cre recombinase, but which is predominantly or only expressed in the heart ventricle and slow-twitch skeletal muscle; is mated with another mouse whose cells comprise a Serca2 ATPase gene modified with loxP recombination sites.

Yet another aspect of the present invention is a method for screening a compound or a mixture of compounds for activity against defective Ca^{2+} handling, comprising the steps of inducing recombination and inactivation of a Serca ATPase gene in a non-human mammal; administrating the compound or mixture to said mammal before and/or after the induced inactivation of the Serca ATPase gene; and observing the effects of the administered compound. The Serca ATPase gene and non-human mammal are as described above and the Serca ATPase gene is inactivated by means of expressing a recombinase, also as described above. Parameters observed might, for

example, be improvement in general condition, alleviation of symptoms, reduced lethality, and improvement of heart contractility.

Another aspect of the invention is a method for screening a compound or a mixture of compounds for activity against heart failure, comprising the steps of inducing recombination and inactivation of a Serca ATPase gene in heart tissue of a non-human mammal; administrating the compound or mixture to said mammal before and/or after the induced inactivation of the Serca ATPase gene; and observing for effects. The Serca ATPase gene and non-human mammal are as described above and the Serca ATPase gene is inactivated by means of expressing a recombinase, also as described above. Parameters observed might, for example, be improvement in general condition, alleviation of symptoms, reduced lethality, and improvement of heart contractility.

The expression "introducing a gene into a mammal" is herein intended to mean any method for producing animals whose cells comprise both a recombinase and a modified Serca gene, both as described above. Examples of such methods are mating, gene therapy and embryo fusion.

Methods, practices and ethics related to gene therapy, mating and embryo fusion, as well as scientific experiments on non-human laboratory mammals are known by a person skilled in the art. Likewise, dosage and administration of a drug or chemical for induction of recombinase expression are known in the art.

Examples of applications of Serca2flox animals

Serca dysfunction and heart failure

The non-human animal all of whose cells comprise a modified Serca ATPase gene can be bred with animal lines carrying the Cre recombinase expressed in the tissue of interest. As for the heart failure research, available animal Cre lines may direct Serca2 gene inactivation specifically in the heart in adult animals, by induction with tamoxifen (Sohal, Nghiem et al. 2001). This particular combination would provide a genetic means of inducing heart failure in a standardized experimental animal model. This model should be applicable for large-scale therapeutic drug screening and development. The major disadvantage of traditional animal models where heart

failure is induced by operative procedures is the difficulty of standardizing operative procedures and their effects on the animals, and the difficulty of large-scale experiments required for therapeutic drug development.

The specificity of Serca2 gene inactivation in live mice is determined by the properties of the Cre recombinase-expressing mouse to which it is mated. For example, mice expressing the Cre recombinase specifically in the heart, brain, liver, lymphoid system or keratinocytes have been made, and can be bred to Serca2flox mice to inactivate the Serca2 gene in those particular organs or tissues of interest.

The major application for Serca2flox mice would be to generate mouse models for human diseases/disorders involving the Serca2 gene by breeding with appropriate recombinase-expressing mice. In the case where the mouse model reflects the phenotype of the human disorder, the mouse model should be a valuable test model for drug development. Inactivation of the Serca2 gene in the heart may induce heart failure, while inactivation of the Serca2 gene in keratinocytes may induce dermatological conditions. Furthermore, it is expected that inactivation of the Serca2 gene in the brain may induce neurological dysfunction, and inactivation of the Serca2 gene in the immune cells may induce immunological dysfunction.

Skeletal muscle Serca dysfunction in heart failure

In congestive heart failure (CHF) patients, skeletal muscle fatigue is a prominent phenotype. Both fast-twitch and slow-twitch muscles seem to become slower, which may be linked to slower intracellular Ca^{2+} cycling (Lunde, Sjaastad et al. 2001; Lunde, Verburg et al. 2002). The neurohumoural stimuli for this change are unknown, but it has been reported that several cytokines are increased in these patients. Thus, the changes seen in skeletal muscles during CHF are in part secondary to inactivity, but may also be a consequence of altered Ca^{2+} metabolism, as seen in the myocardium. Our invention would provide a experimental model for this problem.

Human dermatological disorders

In humans, mutations in the Serca2 gene have been linked with the skin disorders keratosis follicularis (Dariers's disease) and *acrokeratosis verruciformis* (Dhitavat, Macfarlane et al. 2003).

Darier's disease (DD) (keratosis follicularis) in humans is an autosomal dominant skin disorder characterized by loss of adhesion in epidermal cells and abnormal keratinization (Cooper and Burge 2003). Current treatment is rudimentary. However, there is no formal proof that the identified mutations result in loss of function and the observed phenotypes. A mouse model to test for the loss of Serca2 gene function in skin would therefore be necessary to prove the link between genotype and phenotype. Our invention can be bred such that the Serca2 gene is inactivated only in keratinocytes. Such an animal would be the only available animal model for these diseases and for drug development.

Other areas

Ca^{2+} signaling is biologically very important in most tissues, particularly in the brain, immune cells and secretory cells. However, Serca2 function is poorly characterized in these organs and tissues. The Serca2flox mouse could be used to generate mouse models to study Serca2-dependent Ca^{2+} handling functions in these cell types.

A null mutation in the Serca2 gene (*atp2a2*) in mice is embryonic lethal, and demonstrates the necessity of this protein (Periasamy, Reed et al. 1999). Mutations in the human *atp2a2* gene have been linked to dermatological disorders (Cooper and Burge 2003; Dhitavat, Macfarlane et al. 2003), and it is a matter of debate whether some of these mutations also are linked to neuropsychiatric disorders (Jacobsen, Lyons et al. 1999; Jacobsen, Franks et al. 2001; Wang, Yang et al. 2002).

Having now fully described the present invention in some detail by way of illustration and example for purpose of clarity of understanding, it will be obvious to one of ordinary skill in the art that same can be performed by modifying or changing the invention with a wide and equivalent range of conditions, formulations and other parameters thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein

incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

MATERIALS AND METHODS

Animal husbandry and breeeding: Animals were housed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised1996). Mice were housed in M2 cages with Bee Kay bedding (Scanbur BK) in 55% humidity on a 12 h light/dark cycle. Food pellets (mouse + rat standard ex., Scanbur BR) and water was freely available.

Serca2wt/flox mice and MLC-2v-Cre knockin mice (Chen, Kubalak et al. 1998) were backcrossed onto B6/J for n=x and n=XX, respectively before interbreeding. All genotyping was performed on 5 mm tail clips (Torres and Kèuhn 1997) or 1 mm ear punches (Kappler & Marrack Research Laboratory, unpublished protocol) by PCR. Seventeen to 22 week animals of both sexes and genotypes specified in the text were used for basic functional characterization.

Basic in vivo cardiac function: Animals were prepared essencially as described (Woldbaek, Hoen et al. 2002). Briefly, animals were anaestized with i.v. tail injections of 0.2 mL propofol (10 mg/ml) and intubated with a 20-gauge i.v. cannula inserted into the trachea. The animals were ventilated closed chest in the supine position on a rodent ventilator (Model 874 092, B. Braun, Melsungen, Germany) with a mixture of 2% isofluran and 98% oxygen.

Echocardiography:
The transducer was placed gently in the left parasternal position, in principle as described (Sjaastad, Sejersted et al. 2000)2-dimensional, M-mode and Doppler echocardiography was performed with a VIVID 7 echocardiograph (GE Vingmed Ultrasound, Horten, Norway (GE)) using a i13L 13MHz linear array transducer (GE) designed for the examination of small rodents. Data were analyzed with EchoPac PC software (GE).

Haemodynamic pressure measurements:
After echocardiographic measurements, haemodynamic measurements were performed with a 1.4 F Millar micro-tipped pressure transducer catheter (Model SPR-671, Millar Instruments) inserted into the right carotid artery and advanced into the left ventricle (Woldbaek, Tonnessen et al. 2003). Data from 1-2 respiration cycles (10-15 consecutive beats) were used to calculate data including heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressures (LVEDP), the maximal positive (LV+dP/dt) and negative derivatives (LV-dP/dt) of the left ventricular pressure.

Ca2+ ATPase vesicle activity: Vesicular Ca2+ ATPase pumping in and out of SR vesicles was assessed by a fluorometric method.

Ca^{2+} ATPase content: Total Ca^{2+} ATPase in mouse heart left ventricles was measured by an ATP-binding assay in tissue homogenates as described elsewhere (Everts, Andersen et al. 1989; Holt and Christensen 1997).

Cell lines: The mouse embryonic stem cell line ES14.1a (stock at the KTTC, Karolinska Institute, Stockholm, Sweden) was used for genetic manipulations.

Construction of Serca2flox mice: All genetic manipulations were performed using standard molecular biology techniques. The Serca2 (*atp2a2*) gene was cloned from a commercial genomic mouse lambda phage library **(129 SVJ from Stratagene).** A targeting vector, based on pBluescript 2 KS, containing 34 bp loxP sites (5′-ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA T-3′) placed into appropriate positions in *atp2a2* gene fragments and antibiotics selection genes was assembled by standard cloning techniques. The targeting vector was named pSerca2T. All manipulations were confirmed by DNA sequencing (see figure 3). The targeting vector was introduced into mouse embryonic stem cells (ES cells) by standard techniques (electroporation). ES cells carrying homologous recombination events with correctly placed loxP sequences were enriched by antibiotic selection procedures, and identified by Southern hybridization techniques and PCR. The antibiotic selection genes were then excised by a second round of electroporation with a Cre recombinase-expressing plasmid. Correct clones were again identified by Southern blotting and PCR. The final result of the genetic manipulations was that two loxP sites and additional necessary cloning and restriction sites were introduced into 2 separate introns in the *atp2a2* gene. The modification is outlined in figure 4.

Correctly genetically modified ES cells carrying 2 loxP sites in one allele of the *atp2a2* gene were then used to generate genetically modified mice (Serca2flox mice) carrying these loxP recombination sites. The loxP sites are placed such that the *atp2a2* gene is converted into a null allele when the Cre recombinase is appropriately expressed in the organ or tissue of interest.

Subsequent production of all isoforms of Serca2 Ca^{2+}ATPase protein is reduced or eliminated in the tissue of interest

In vivo basic performance: Heart rhythms and locomoter activity were evaluated by telemetric analysis.

Mice: The mouse line was backcrossed onto the B6/J background (N10), and bred to homozygosity.

Northern blotting: Gene expression in heart left ventricles was analyzed basically as described (Semb, Lunde et al. 1998). Poly A^+ RNA was captured onto Dynabeads oligo$(dT)_{25}$ according to the manufacturers protocol (Dynal AS, Norway). Samples (10 µg mRNA) were size-fractionated on 1% agarose gels and. Filters were probed with cDNA framents for for Serca2 (pRH39, EcoRI 2.2 kb insert)(Lompre, de la Bastie et al. 1989), ANP (pGANP-1, PstI 650 bp insert) (Glembotski, Oronzi et al. 1987) and GAPDH (1.3 kb insert) (a kind gift from Hans Prydz).

PCR detection: Different alleles of the Serca2 gene (Serca2 wt, flox or deletion) and the MLC-2v gene (wt and cre knock-in) were detected by standard PCR reactions with annealing temperature of 55 °C for 25-30 cycles and Amplitaq Gold on an ABI 9600 thermocycler (Applied Biosystems). Primers were as follows:

OL84	5′- cca agg aag atg gct gac c - 3′
OL85	5′- cat cga cgc ctc ata aat cc - 3′
OL86	5′- tct tca taa cac acg cca att t - 3′
OL87	5′- ccc ttt gct gcc aat taa cta tt - 3′
OL88	5′- acc tct agg ggt ctc gaa tca - 3′
OL102	5′- aag ttg aat aac cgg aaa tgg ttt - 3′
OL103	5′- tgt tat aag caa tcc cca gaa atg - 3′
OL104	5′- agg ctc ctc gaa ctc tcc ag - 3′
OL105	5′- gta aga gag ctt ccc tcc tcc tt - 3′

Primer combinations and PCR amplification product sizes are given below:

Position	Primer pair	PCR product
Serca2 intron 1	OL84 / OL85	wt: 247 bp
		flox: 419 bp
Serca2 intron 1	OL88 / OL85	flox: 318 bp
Serca2 intron 3	OL86 / OL87	wt: 282 bp
		flox: 381 bp
Serca2 deletion	OL88 / OL87	324 bp
Cre (knockin)	OL102 / OL103	340 bp
MLC-2v wt	OL104 / OL105	183 bp

Statistical analysis: The data are expressed as mean values +/-S.E.M. Statistical significance was calculated using Student's unpaired t-test (Graphpad Prism, 4.01). P values <0.05 were considered significant.

Tissue material: Immediately after conclusion of the *in vivo* measurements, the mice were sacrificed with excision of heart, lung, liver, kidney, spleen, soleus and extensor digitorum longus (EDL) muscles. Hearts and lungs were blotted dry and immediately weighed. All organs were flash frozen in liquid nitrogen.

Western blotting: Mouse tissues were homogenized in ice-cold buffer (210 mM sucrose, 2mM EGTA, 40mM NaCl, 30mM Hepes, 5mM EDTA, pH 7.4) supplemented with a protease inhibitor cocktail (Complete EDTA-free, Roche Diagnostics, Mannheim, Germany) essencially as described (Ploug, Wojtaszewski et al. 1993). Homogenization buffer was also supplemented with phosphatase inhibitors (phosphatase inhibitor cocktail 1, Sigma) where needed. SDS was added to 1% final concentration, and the lysate was incubated for 15 minutes at room temperature before aliquoting and freezing at –80 °C. Protein content was measured by a bicinchoninic acid assay (Pierce 23235) using BSA as standard protein. Samples were electrophoresed in SDS-PAGE gels (8% or 15%) and transferred to 0.45µm PVDF membranes by standard procedures. Membranes were blocked in 5% skim milk in TBS-T (Amersham Life Sciences) or in an alternative BPPT blocking solution (5% BSA, 1% polyvinylpyrrolidone-10, 1% polyethyleneglycol, MW 3500, 0.2% Tween 20 in 2xTBS) (NanoTools Antikörpertechnik, info@nanotools.de) where indicated. The following antibodies were used for protein detection: monoclonal antibodies against Serca2a (MA3-919) , phospholamban PLB (MA3-922) (all Affinity Bioreagents), polyclonal anti-Serca2b antiserum(Campbell, Wuytack et al. 1993) (kind

gift from Frank Wuytack), anti-NCX1 (Thomas, Sjaastad et al. 2003) (a kind gift from Marion Thomas), anti-phospho-Ser16-PLB and anti-phospho-Thr17-PLB (both Badrilla). Blots were incubated with appropriate HRP-conjugated sheep anti-mouse IgG or donkey anti-rabbit IgG secondary antibodies (Amersham Biosciences) and developed with ECL or ECLplus reagents (Amersham Biosciences). Images were acquired in a LAS-1000 CCD detection system (Fuji film). All membranes were post-immunoblotting stained with Coomassie Brilliant Blue R250, and regions in each lane were used for normalization of protein loading on the membrane.

EXAMPLES

Example 1: Generation of cardiac-specific Serca2$^{wt/flox}$ heterozygous mice

Serca2flox mice were intercrossed with MLC-2v Cre knock-in mice (Chen, Kubalak et al. 1998; Minamisawa, Gu et al. 1999) in which the Cre recombinase is expressed in the heart ventricle and in slow-twitch skeletal muscle starting at embryonic day E8 with no detectable basal expression in other tissues. The Serca2$^{wt/flox}$ and MLC-2v Cre knock-in mice were at generation N3 and N3 onto the B6/J background before interbreeding. In the final breeding between Serca2$^{flox/flox}$ and Serca2$^{wt/flox}$ MLC2v$^{wt/cre}$ animals, we were unable to obtain offspring with the expected homozygous "cardiac knockout" genotype (Serca2$^{flox/flox}$ MLC2v$^{wt/cre}$) (table 1). More than 100 offspring were genotyped. In the six mating pairs (with alternation of parental genotypes), we could detect offspring with only 2 of the 4 expected genotypes (Serca2$^{flox/flox}$ MLC2v$^{wt/wt}$ and Serca2$^{wt/flox}$ MLC2v$^{wt/cre}$). Since litter sizes were normal and the wild type genotype also was missing, the genotype distribution indicated that the Serca2 and MLC2-v gene loci did not segregate freely. The breeding result could be explained by a lack of chromosomal crossover between the Serca2 (*atp2a2*) and MLC-2v (*myl2*) loci. During this breeding period, the MLC-2v gene was assigned a chromosomal position in close proximity to Serca2 on mouse chromosome 5, cytoband F (approx. distance 630 000 nt). In conclusion, the linkage between the Serca2 (atp2a2) and MLC-2*v* (myl2) genes on chromosome 5 is for practical purposes 100%.

Example 2: Characterization of cardiac-specific Serca2$^{wt/flox}$ heterozygous mice

Specific gene inactivation

The tissue specificity of the Serca2 gene inactivation was confirmed by PCR analysis of DNA from mouse tissues (Fig. 5). In Serca2$^{wt/flox}$ MLC-2v$^{wt/cre}$ double heterozygous mice, deletion of one copy of the Serca2 gene was detected only in the heart and in slow-twitch skeletal muscle (soleus). No Serca2 deletion PCR product was detected in EDL (fast-twitch) muscle, liver, lung, kidney or spleen. No excision was detected in control littermates (Serca2$^{wt/flox}$ MLC-2v$^{wt/wt}$). This result was expected from the expression of Cre recombinase from the MLC-2v gene locus in this particular cross.

Animal physiology

The animal age, heart, lung and body weights were measured in Serca2$^{wt/flox}$ MLC-2v$^{wt/cre}$ double heterozygous mice and in control littermates (Serca2$^{wt/flox}$ MLC-2v$^{wt/wt}$) (Table 2). In total, hearts and organs were examined from 71 animals. There were no gross anatomical differences. Overall, there was no significant difference between the two genotypes in animal size, heart and lung weight, or in the ratios of heart weight/body weight or lung weight/body, indicative of no overt disease in the double heterozygous animals. As expected, females had significantly lower body weight, heart weight, and lung weight than males. However, no significant differences were detected between the genotypes within each sex.

Serca2 expression in cardiac heterozygous Serca2 animals

The amount of functional Ca^{2+}ATPase enzyme in hearts from both genotypes was measured by an ATP-binding assay (table 5). In Serca2$^{wt/flox}$ MLC-2v$^{wt/cre}$ double heterozygous mice, the content of ATPase in the heart was reduced by 32% in 11 week old mice (n=2 for each group), and by 28% in 19-week old mice compared with control Serca2$^{flox/flox}$ MLC-2v$^{wt/wt}$ littermates .

The protein content of both Serca2a and Serca2b isoforms were examined in both left ventricle and in slow-twitch soleus muscle by Western blotting with isoform-specific antibodies. In the left ventricle, Serca2a was reduced by 28% and 26% in double heterozygous Serca2$^{wt/flox}$ MLC-2v$^{wt/cre}$ mice (figure 7, panel A) compared with control Serca2$^{flox/flox}$ MLC-2v$^{wt/wt}$ littermates. A parallel decrease in cardiac Serca2b protein was also detected, 49% in double heterozygotes compared to control animals (panel b). In the soleus muscle, Serca2a and Serca2b protein ws reduced by 25% and 35% in the double heterozygotes compared to control animals (figure 7, panel B). In other tissues such as lung, spleen, liver and kidney, there was no decrease in Serca2b expression in the double heterozygous animals relative to controls (figure 7, panel B, lower part). Taken together, the amount Serca2a and Serca2b proteins were reduced specifically in the "cardiac Serca2 heterozygous" animals in the heart and in slow-twitch soleus muscle, but not in other tissues.

In vivo heart function

Basal *in vivo* heart function was first assessed by echocardiography (table 3). Overall heart dimensions, aorta dimensions, flow rates and cardiac output was not significantly different between the cardiac heterozygous Serca2 ($Serca2^{wt/flox}$ $MLC\text{-}2v^{wt/cre}$) and control animals ($Serca2^{wt/flox}$ $MLC\text{-}2v^{wt/wt}$). There were no indications of cardiac dysfunction in either genotype.

Haemodynamic pressure measurements were then sequentially performed on each animal with a pressure transducer inserted into the left ventricle. Again, no differences were observed between the two genotypes, nor any indications of cardiac dysfunction (table 4).

Atrial natriuretic factor is readily induced in stressed, hypertrophic or dysfunctional hearts. No differences in ANP expression between the two genotypes was detected, arguing against the hearts being in a stressed state at a subclinical level (figure 6).

Compensatory mechanisms in Serca2 expression in cardiac heterozygous animals

The total level of phospholamban was unchanged, as evaluated by PLB monomers under reducing conditions (figure 8). Likewise, there was no shift in Ser-16 or Thr-17 phosphorylated monomers in double heterozygous animals relative to controls. However, there seems to be shift in the content of pentameric PLB protein in the heterozygotes compated with flox controls.

In the heart, NCX1 RNA expression was increased by 40% (figure 8, panel C). In EDL and soleus muscle, there was no detectable NCX1 immunoreactivity. This is in keeping with data from the Serca2 heterozygous mouse (Periasamy, Reed et al. 1999), and provides an explanation for compensatory pathway for removing Ca^{2+} from the cytosol out of the cell via NCX1, since the expression level of Serca2, and removal of Ca^{2+} into the SR, is reduced

Summary

In our model, the gene inactivation occurred only in the tissue of interest, as demonstrated in this particular test breeding of Serca2flox mice of the invention with MLC-2v Cre mice. Serca2a was partially inactivated in the heart and in slow skeletal muscle of $Serca2^{wt/flox}$ $MLC\text{-}2v^{wt/cre}$ double heterozygous mice as shown by PCR, ATPase and protein measurements.

In conclusion, the invention can be used to generate standardized animal models with defective Ca^{2+} handling in the tissue of organ of interest, and thus be useful in biomedical research and therapeutic drug development.

Example 3: Generation of cardiac tamoxifen-inducible Serca2 knockout mice

We were, as described in Example 1, unable to obtain cardiac Serca2 knockout mice by breeding $Serca2^{flox}$ mice with MLC-2v-Cre mice due to locus linkage. $Serca2^{flox}$ mice bred to transgenic MerCreMer mice, which express the Cre recombinase in the heart under control of the α-myosin heavy chain promoter (Sohal, Nghiem et al. 2001). In addition, the presence of Cre recombinase in the nucleus, and thereby activity, is controlled by tamoxifen.

Example 4: Induction of Serca2 inactivation

Cardiac Serca2 knockout mice are produced by injection of $Serca2^{flox/flox}$ MerCreMer with tamoxifen as described (Sohal, Nghiem et al. 2001). The animals are characterized as the heterozygous animals in Examples 1 to 2. In addition, the forward and reverse mode functions of Serca2 ATPase in cardiac SR vesicles will be measured.

One predicted phenotype of this cardiac restricted Serca2 knockout mouse can be full compensation because other proteins are able to sufficiently handle the Ca^{2+} movements in the cardiomyocyte (e.g. NCX1). The other extreme phenotype may be sudden death due to a collapse in the electrochemical balance in the cardiomyocytes (arrhythmic phenotype). In this respect, the animals are evaluated by ECG to detect arrhythmic activity. We anticipate that there will be a time window in which Serca2 protein progressively is reduced. The heart will first have sufficient time to develop compensatory mechanisms, but then progress into a failing state as the Serca2 function is severely reduced. The heart may display a hypertrophic phenotype with activation of cytokine systems and other signal transduction pathways. This model would then mimic the compensated and failing stages of heart failure in humans. Given the latter scenario, the endpoint will be death. This is a "hard" endpoint ideal for the invention's proposed use as a tool for developing improved drugs and therapies against heart failure. Also, another feasible feature will be the extended time window which will permit time for conducting the experiment as well as monitoring.

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Tables:

Table 1

Genotype distribution of breeding results for Serca2flox and MLC-2*v*-Cre knock-in mice.

Cross: Parental genotypes $Serca2^{wt/flox}$ $MLC\text{-}2v^{wt/cre}$ X $Serca2^{flox/flox}$ $MLC\text{-}2v^{wt/wt}$

Offspring	Genotype	n=101	%	Expected % (free segregation)	Expected % (no crossover)
Serca2 Flox heterozygous control	$Serca2^{wt/flox}$ $MLC\text{-}2v^{wt/wt}$	0	0	25	0
Serca2 Flox homozygous control	$Serca2^{flox/flox}$ $MLC\text{-}2v^{wt/wt}$	44	44	25	50
Cardiac Serca2 heterozygous	$Serca2^{wt/flox}$ $MLC\text{-}2v^{wt/cre}$	56	55	25	50
Cardiac Serca2 knockout	$Serca2^{flox/flox}$ $MLC\text{-}2v^{wt/cre}$	0	0	25	0

Table 2

Body and organ weights for Serca2 cardiac heterozygous and control mice. Age, body, heart and lung weights of control and heterozygous animals (Mean ± SEM).

By genotype	FF	n	FC	n
Body weight (g)	28.0 ± 0.7	40	27.9 ± 0.9	31
Heart weight (mg)	105.9 ± 3.1	40	105.5 ± 4.0	31
Lung weight (mg)	155.6 ± 4.0	35	148.2 ± 3.5	30
Heart weight / Body Weight (mg/g)	3.8 ± 0.1	40	3.8 ± 0.1	31
Lung weight /Body weight (mg(g)	5.6 ± 0.2	35	5.4 ± 0.2	30

By sex	M	n	F	n
Body weight (g)	33.2 ± 1.2	19	26.0± 0.3 *	52
Heart weight (mg)	127.9 ± 5.6	19	97.6 ± 1.6 $	52
Lung weight (mg)	165.6 ± 5.3	19	146.7 ± 2.8 #	46
Heart weight / Body Weight (mg/g)	3.9 ± 0.1	19	3.8 ± 0.1	52
Lung weight /Body weight (mg(g)	5.1 ± 0.2	19	5.7 ± 0.1 &	46

* p<0.0001, $ p<0.0001, # p=0.0009, & p=0.021

Males by genotype	M FF	n	M FC	n
Body weight (g)	33.4 ± 1.7	10	32.9± 2.0	9
Heart weight (mg)	126.3 ± 8.4	10	129.7 ± 7.9	9
Lung weight (mg)	170.1 ± 10.9	8	161.8 ± 6.0	9
Heart weight / Body Weight (mg/g)	3.8 ± 0.2	10	4.0 ± 0.2	9
Lung weight /Body weight (mg(g)	5.2 ± 0.4	10	5.0 ± 0.2	9

Females by genotype	F FF	n	F FC	n
Body weight (g)	26.2 ± 0.5	30	25.9 ± 0.5	22
Heart weight (mg)	99.1 ± 2.0	30	95.6 ± 2.5	22
Lung weight (mg)	150.2 ± 4.0	30	142.4 ± 3.6	21
Heart weight / Body Weight (mg/g)	3.8 ± 0.1	30	3.7 ± 0.1	22
Lung weight /Body weight (mg(g)	5.8 ± 0.2	25	5.5 ± 0.2	21

FF = $Serca2^{flox/flox}$ M = males

FC = Serca2$^{wt/flox}$ MLC-2v$^{wt/cre}$ F= females

Table 3

Heart dimensions and flow parameters

Echocardiographic data

		FF	FC
	n	14	14
M-mode			
	IVSd (mm)	0.97 ± 0.02	0.99 ± 0.02
	IVSs (mm)	1.56 ± 0.04	1.59 ± 0.04
	IVS thickening (%)	61 ± 3	61 ± 4
	LVDd (mm)	3.76 ± 0.08	3.60 ± 0.07
	LVDs (mm)	2.36 ± 0.10	2.16 ± 0.09
	FS (%)	37 ± 2	40 ± 2
	PWd (mm)	0.89 ± 0.02	0.88 ± 0.03
	PWs (mm)	1.34 ± 0.05	1.34 ± 0.06
	PW thickening (%)	52 ± 6	53 ± 5
	PWSV ($cm\ s^{-1}$)	2.12 ± 0.11	2.02 ± 0.08
	PWRV ($cm\ s^{-1}$)	2.28 ± 0.14	2.5 ± 0.13
2-D			
	IVSd (mm)	1.01 ± 0.03	0.99 ± 0.03
	IVSs (mm)	1.42 ± 0.03	1.44 ± 0.02
	IVS thickening (%)	44 ± 3	44 ± 2
	LVDd (mm)	3.62 ± 0.12	3.50 ± 0.10
	LVDs (mm)	2.49 ± 0.11	2.37 ± 0.09
	FS (%)	31 ± 1	32 ± 1
	PWd (mm)	0.94 ± 0.02	0.93 ± 0.03
	PWs (mm)	1.34 ± 0.05	1.35 ± 0.04
	PW thickening (%)	45 ± 3	50 ± 3
	Aorta (mm)	1.38 ± 0.03	1.36 ± 0.02
Doppler			
	Peak mitral flow ($m\ s^{-1}$)	0.68 ± 0.03	0,72 ± 0,03
	Peak LVOT flow ($m\ s^{-1}$)	1.28 ± 0.06	1.24 ± 0,06
	Peak RVOT flow ($m\ s^{-1}$)	0.59 ± 0.03	0.54 ± 0.03
	VTI in LVOT	4.44 ± 0.21	4.23 ± 0,24
	CO in LVOT ($ml\ min^{-1}$)	31.5 ± 2.3	31.7 ± 2,1

FF, $Serca2^{flox/flox}$; FC, $Serca2^{wt/flox}$ $MLC\text{-}2v^{wt/cre}$; IVSd/s, inter-ventricular septum thickness in diastole respectively systole; LVDd/s, left ventricular diameter in diastole respectively systole; FS, fractional shortening in LVD; PWd/s, posterior wall thickness in diastole respectively systole; LAD, left atrial diameter; PWSV, posterior wall shortening velocity; PWRV, posterior wall relaxation velocity; LVOT, left ventricular outlet tract; RVOT, right ventricular outlet tract; CO, cardiac output; VTI, velocity time integral

All values are mean ± SEM

Table 4: Haemodynamic pressure measurements.

Pressure data

Genotype	FF			FC		
n	14			16		
HR (beats/min)	551	±	25	538	±	19
LVSP (mm Hg)	115,5	±	5,0	111,6	±	5,0
LVEDP (mm Hg)	5,0	±	0,3	6,3	±	1,5
LVdP/dtmax	9772	±	811	8402	±	749
LVdP/dtmin	-10020	±	681	-8840	±	700

Genotypes: (Control) FF = $Serca2^{flox/flox}$ (Cardiac heterozygous) FC = $Serca2^{wt/flox}$ $MLC\text{-}2v^{wt/cre}$

Table 5

Reduction of functional cardiac Ca^{2+} ATPase protein content in test model.

Measurement of cardiac Ca^{2+} ATPase content in control and heterozygous animals (Mean ± SEM).

Age (weeks)	nmol Ca^{2+} ATPase / g protein		Reduction ATPAse content (%)
	FF	FC	
11	47 (n=2)	26 (n=2)	32
19	32 ± 3 (n=7)	23 ± 1 * (n=5)	28

* $p<0.0005$

FF = $Serca2^{flox/flox}$

FC = $Serca2^{wt/flox}$ $MLC\text{-}2v^{wt/cre}$

We claim:

1. A transgenic non-human mammal all of whose cells comprise a Serca ATPase gene modified by inserted recombination sites.

2. The mammal of claim 1 comprising one or several copies of the modified Serca ATPase gene.

3. The mammal of claim 1, wherein the Serca ATPase gene is a Serca2 ATPase gene.

4. The mammal of claim 1, wherein the recombination sites are of heterogenous origin.

5. The mammal of claim 4, wherein the heterogenous recombination sites are of non-mammalian origin.

6. The mammal of claim 5, wherein the recombination sites comprise loxP recombination sites.

7. The mammal of claim 1 all of whose cells further comprise a gene encoding a heterogenous recombinase.

8. The mammal of claim 7, wherein the heterogenous recombinase is of non-mammalian origin.

9. The mammal of claim 8, wherein the recombinase is a Cre recombinase.

10. The mammal of claim 7, wherein expression of the recombinase encoding gene is controlled by a regulatory nucleic acid sequence.

11. The mammal of claim 10, wherein the regulatory nucleic acid sequence is inducible.

12. The mammal of claim 7, wherein expression of the recombinase gene is tissue-specific.

13. The mammal of claim 12, wherein expression of the recombinase gene occurs in heart tissue.

14. The mammal of claim 1, wherein the mammal is a rodent.

15. The mammal of claim 14, wherein the rodent is a mouse.

16. A eukaryotic cell comprising a Serca ATPase gene modified by inserted recombination sites.

17. The cell of claim 16 comprising one or several copies of the modified Serca ATPase gene.

18. The cell of claim 16, wherein the Serca ATPase gene is a Serca2 ATPase gene.

19. The cell of claim 16, wherein the recombination sites are of heterogenous origin.

20. The cell of claim 19, wherein the heterogenous recombination sites are of non-mammalian origin.

21. The cell of claim 20, wherein the recombination sites comprise loxP recombination sites.

22. The cell of claim 16 further comprising a gene encoding a heterogenous recombinase.

23. The cell of claim 22, wherein the heterogenous recombinase is of non-mammalian origin.

24. The cell of claim 23, wherein the recombinase is a Cre recombinase.

25. The cell of claim 22, wherein expression of the recombinase encoding gene is controlled by a regulatory nucleic acid sequence.

26. The cell of claim 25, wherein the regulatory nucleic acid sequence is inducible.

27. The cell of claim 16, wherein the cell is of mammalian origin.

28. The cell of claim 27, wherein the cell is of non-human mammalian origin.

29. The cell of claim 28, wherein the cell is of rodent origin.

30. The cell of claim 39, wherein the cell is of mouse origin.

31. The cell of anyone of claims 16-30, wherein said cell is an embryonic cell.

32. The cell of anyone of claims 16-30, wherein said cell is a cardiomyocyte.

33. A gene encoding a Serca ATPase modified by inserted recombination sites.

34. The gene of claim 33, wherein the Serca ATPase is a Serca2 ATPase

35. The gene of claim 33, wherein the recombination sites are of heterogenous origin.

36. The gene of claim 35, wherein the heterogenous recombination sites are of non-mammalian origin.

37. The gene of claim 36, wherein the recombination sites comprise loxP recombination sites.

38. The gene of claim 33, wherein said gene has a nucleic sequence substantially as set forth in SEQ ID 1.

39. A vector comprising the gene of claim 33.

40. The vector of claim 39, wherein the vector is based on pBluescript 2 KS.

41. A method for inducing defective Ca^{2+} handling in a non-human mammal, comprising the steps of inducing recombination and inactivation of a Serca ATPase gene.

42. The method of claim 41, wherein the Serca ATPase gene is a Serca2 ATPase gene.

43. The method of claim 41, wherein the Serca gene is inactivated in heart tissue.

44. The method of claim 41, wherein said mammal is the mammal of anyone of claims 7, 8, 9, 10, 11, or 12.

45. A method for inducing heart failure in non-human mammals, comprising the steps of inducing recombination and inactivation of a Serca ATPase gene in heart tissue.

46. The method of claim 45, wherein the Serca ATPase gene is a Serca2 ATPase gene.

47. The method of claim 45, wherein said mammal is the mammal of claim 13.

48. A method for screening a compound or a mixture of compounds for activity against defective Ca^{2+} handling, comprising the steps of inducing recombination and inactivation of a Serca ATPase gene in a non-human mammal; administrating the compound or mixture to said mammal before and/or after the induced inactivation of the Serca ATPase gene.

49. The method of claim 48, wherein the Serca ATPase gene is a Serca2 ATPase gene.

50. The method of claim 48, wherein the Serca gene is inactivated in heart tissue.

51. The method of claim 48, wherein said mammal is the mammal of anyone of claims 7, 8, 9, 10, 11, or 12.

52. A method for screening a compound or a mixture of compounds for activity against heart failure, comprising the steps of inducing recombination and inactivation of a Serca ATPase gene in heart tissue of a non-human mammal; administrating the compound or mixture to said mammal before and/or after the induced inactivation of the Serca ATPase gene.

53. The method of claim 52, wherein the Serca ATPase gene is a Serca2 ATPase gene.

54. The method of claim 52, wherein said mammal is the mammal of claim 13.

ABSTRACT

The present invention relates to a non-human mammal in which all cells contain genetic modifications in a Ca^{2+} handling Serca ATPase gene, more particularly the Serca2 ATPase. Defective Ca^{2+} handling is induced in live animals by introducing genetic elements which direct the timing and specificity of the gene inactivation to the organ or tissue of interest; e.g. cardiac muscle cells. The primary application is to provide a standardized and reproducible animal model for heart failure or other human diseases, in which the defective Ca^{2+} handling function by the Serca gene can be manipulated in live animals.. This is the only existing animal to date with such genetic modifications for this class of Ca^{2+} handling proteins.

Figure 1 **Sequence alignment of mouse Serca 1, 2 and 3 protein.**

```
Serca1a    1 MEAAHSKSTEECLSYFGVSETTGLTPDQVKRHLEKYGPNELPAEEGKSLWELVVEQFEDL
Serca2a    1 ..N..T.TV..V.GH...N.S...SLE...KLK.RW.S.........T.L...I......
Serca2b    1 ..N..T.TV..V.GH...N.S...SLE...KLK.RW.S.........T.L...I......
Serca3a    1 ..E..LL.AADV.RR.S.TAEG..SLE..TDAR.R.......T.................
Serca3b    1 ..E..LL.AADV.RR.S.TAEG..SLE..TDAR.R.......T.................
Serca3c    1 ..E..LL.AADV.RR.S.TAEG..SLE..TDAR.R.......T.................

Serca1a   61 LVRILLLAACISFVLAWFEEGEETVTAFVEPFVILLILIANAIVGVWQERNAENAIEALK
Serca2a   61 ........................I.............V.....................
Serca2b   61 ........................I.............V.....................
Serca3a   61 .........LV.............T......L..M...V..............S......
Serca3b   61 .........LV.............T......L..M...V..............S......
Serca3c   61 .........LV.............T......L..M...V..............S......

Serca1a  121 EYEPEMGKVYRADRKSVQRIKARDIVPGDIVEVAVGDKVPADIRILSIKSTTLRVDQSIL
Serca2a  121 ...........Q..........K.........I...........LT..............
Serca2b  121 ...........Q..........K.........I...........LT..............
Serca3a  121 .........I.S...G....R.....................L.LIE.............
Serca3b  121 .........I.S...G....R.....................L.LIE.............
Serca3c  121 .........I.S...G....R.....................L.LIE.............

Serca1a  181 TGESVSVIKHTDPVPDPRAVNQDKKNMLFSGTNIAAGKAVGIVATTGVSTEIGKIRDQMA
Serca2a  181 ....................................M.V.VA...N........E.V
Serca2b  181 ....................................M.V.VA...N........E.V
Serca3a  181 .......T....AI.....................S...L.VAVA..LQ..L....S...
Serca3b  181 .......T....AI.....................S...L.VAVA..LQ..L....S...
Serca3c  181 .......T....AI.....................S...L.VAVA..LQ..L....S...

Serca1a  241 ATEQDKTPLQQKLDEFGEQLSKVISLICVAVWLINIGHFNDPVHGGSWFRGAIYYFKIAV
Serca2a  241 ....ER.....................I...I.............I...........
Serca2b  241 ....ER.....................I...I.............I...........
Serca3a  241 .V.PER....R......R...HA..V......V......A..A.....L...V.......
Serca3b  241 .V.PER....R......R...HA..V......V......A..A.....L...V.......
Serca3c  241 .V.PER....R......R...HA..V......V......A..A.....L...V.......

Serca1a  301 ALAVAAIPEGLPAVITTCLALGTRRMAKKNAIVRSLPSVETLGCTSVICSDKTGTLTTNQ
Serca2a  301 ..................................................
Serca2b  301 ..................................................
Serca3a  301 ........................R.........................
Serca3b  301 ........................R.........................
Serca3c  301 ........................R.........................

Serca1a  361 MSVCKMFIIDKVDGDVCSLNEFSITGSTYAPEGEVLKNDKPVRAGQYDGLVELATICALC
Serca2a  361 ....R...L...E..T.............I...Q.D....KCH.............
Serca2b  361 ....R...L...E..T.............I...Q.D....KCH.............
Serca3a  361 ....R..VVAEAEAGT.R.H..T.S.T..T.....RQGEQ...C..F.........
Serca3b  361 ....R..VVAEAEAGT.R.H..T.S.T..T.....RQGEQ...C..F.........
Serca3c  361 ....R..VVAEAEAGT.R.H..T.S.T..T.....RQGEQ...C..F.........

Serca1a  421 NDSSLDFNETKGVYEKVGEATETALTTLVEKMNVFNTEVRSLSKVERANACNSVIRQLMK
Serca2a  421 ...A..Y..A.............C........D..LKG...I.........K....
Serca2b  421 ...A..Y..A.............C........D..LKG...I.........K....
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Serca3a  421 ...A..Y..A.................C........D.DLKG..R....G......K...R
Serca3b  421 ...A..Y..A.................C........D.DLKG..R....G......K...R
Serca3c  421 ...A..Y..A.................C........D.DLKG..R....G......K...R
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Serca1a  481 KEFTLEFSRDRKSMSVYCSPAKSSRAAVGNKMFVKGAPEGVIDRCNYVRVGTTRVPLTGP
Serca2a  481 .................T.N.P..TSMS-.............THI...S.K..M.PG
Serca2b  481 .................T.N.P..TSMS-.............THI...S.K..M.PG
Serca3a  481 .................T.TRADPKVQ.S.........S..E..SS....SRTA..STT
Serca3b  481 .................T.TRADPKVQ.S.........S..E..SS....SRTA..STT
Serca3c  481 .................T.TRADPKVQ.S.........S..E..SS....SRTA..STT

Serca1a  541 VKEKIMSVIKEWGTGRDTLRCLALATRDTPPKREEMVLDDSAKFMEYEMDLTFVGVVGML
Serca2a  540 ..Q......R...S.S..........H.N.L.....H.E...N.IK..TN.....C....
Serca2b  540 ..Q......R...S.S..........H.N.L.....H.E...N.IK..TN.....C....
Serca3a  541 SR.H.LAK.RD..S.S..............RK.D.H...CSR.VQ..T......C....
Serca3b  541 SR.H.LAK.RD..S.S..............RK.D.H...CSR.VQ..T......C....
Serca3c  541 SR.H.LAK.RD..S.S..............RK.D.H...CSR.VQ..T......C....

Serca1a  601 DPPRKEVTGSIQLCRDAGIRVIMITGDNKGTAIAICRRIGIFSENEEVTDRAYTGREFDD
Serca2a  600 ....I..AS.VK...Q..............V.........GQD.D..SK.F......E
Serca2b  600 ....I..AS.VK...Q..............V.........GQD.D..SK.F......E
Serca3a  601 ....P..AAC.TR.SR.....V..........V.....L...GDT.D.LGK.........
Serca3b  601 ....P..AAC.TR.SR.....V..........V.....L...GDT.D.LGK.........
Serca3c  601 ....P..AAC.TR.SR.....V..........V.....L...GDT.D.LGK.........

Serca1a  661 LPLAEQREACRRACCFARVEPSHKSKIVEYLQSYDEITAMTGDGVNDAPALKKAEIGIAM
Serca2a  660 .SPSA..D..LN.R..............F...F...................S.......
Serca2b  660 .SPSA..D..LN.R..............F...F...................S.......
Serca3a  661 .SPEQ..Q...T.R.......A...R...N...FN.........................
Serca3b  661 .SPEQ..Q...T.R.......A...R...N...FN.........................
Serca3c  661 .SPEQ..Q...T.R.......A...R...N...FN.........................

Serca1a  721 GSGTAVAKTASEMVLADDNFSTIVAAVEEGRAIYNNMKQFIRYLISSNVGEVVCIFLTAA
Serca2a  720 ..................................................................
Serca2b  720 ..................................................................
Serca3a  721 ........S.A....S....AS.....................................I
Serca3b  721 ........S.A....S....AS.....................................I
Serca3c  721 ........S.A....S....AS.....................................I

Serca1a  781 LGLPEALIPVQLLWVNLVTDGLPATALGFNPPDLDIMDRPPRSPKEPLISGWLFFRYMAI
Serca2a  780 ..F.................................NK...N.............L..
Serca2b  780 ..F.................................NK...N.............L..
Serca3a  781 ....................................EK...N.R.A.........L..
Serca3b  781 ....................................EK...N.R.A.........L..
Serca3c  781 ....................................EK...N.R.A.........L..

Serca1a  841 GGYVGAATVGAAAWWFLYAEDGPHVSYHQLTHFMQCTEHNPEFDGLDCEVFEAPEPMTMA
Serca2a  840 .C.............IA.DG..R..FY..S..L..K.D..D...V..AI..S.Y.....
Serca2b  840 .C.............IA.DG..R..FY..S..L..K.D..D...V..AI..S.Y.....
Serca3a  841 .V...L...A..T.....DAE..Q.TFY..RN.LK.S.D..L.A.I..K...SRF.T...
Serca3b  841 .V...L...A..T.....DAE..Q.TFY..RN.LK.S.D..L.A.I..K...SRF.T...
Serca3c  841 .V...L...A..T.....DAE..Q.TFY..RN.LK.S.D..L.A.I..K...SRF.T...

Serca1a  901 LSVLVTIEMCNALNSLSENQSLLRMPPWVNIWLLGSICLSMSLHFLILYVDPLPMIFKLR
Serca2a  900 ..........................E....V.............E...L..QIT
Serca2b  900 ..........................E....V.............E...L..QIT
Serca3a  901 ..............V...........L.P....AVVM..A.....L.P...L..QVT
Serca3b  901 ..............V...........L.P....AVVM..A.....L.P...L..QVT
Serca3c  901 ..............V...........L.P....AVVM..A.....L.P...L..QVT
```

```
Serca1a   961 ALDFTQWLMVLKISLPVIGLDELLKFIARNYLEG
Serca2a   960 P.NL.............LM..T...V......QPAILE
Serca2b   960 P.NL.............LM..T...V......QPGKECVQPATKSSCSLSACTDGISWP
Serca3a   961 P.SGR..GV..QM.....L...A..YLS..HMDEKKDLK
Serca3b   961 P.SGR..GV..QM.....L...A..YLS..HMD.VLGTFMQARSRQLPTTSRTPYHTGKK
Serca3c   961 P.SGR..GV..QM.....L...A..YLS..HMD.VLGTFMQARSRQLPTTSRTPYHTGLA


Serca2b 1020 FVLLIMPLVVWVYSTDTNFSDMFWS
Serca3b 1021 GPEVNPGSRGESPVWPSD
Serca3c 1021 SWKKRT
```

Figure 2 **Sequence similarity of Serca2 proteins in mammalian species**

```
Mouse_2a      1 MENAHTKTVEEVLGHFGVNESTGLSLEQVKKLKERWGSNELPAEEGKTLLELVIEQFEDL
Mouse_2b      1 ............................................................
Rat_2b        1 ............................................................
Rat_2a        1 ............................................................
Dog_2a        1 ............................................................
Cat_2a        1 ............Y...............................................
Pig_2a        1 ............................................................
Pig_2b        1 ............................................................
Human_2b      1 ............................................................
Human_2c      1 ............................................................
Human_2a      1 ............................................................
Rabbit_2a     1 ............................................................
Rabbit_2b     1 ............................................................

Mouse_2a     61 LVRILLLAACISFVLAWFEEGEETITAFVEPFVILLILVANAIVGVWQERNAENAIEALK
Mouse_2b     61 ............................................................
Rat_2b       61 ............................................................
Rat_2a       61 ............................................................
Dog_2a       61 ............................................................
Cat_2a       61 ............................................................
Pig_2a       61 ............................................................
Pig_2b       61 ............................................................
Human_2b     61 ............................................................
Human_2c     61 ............................................................
Human_2a     61 ............................................................
Rabbit_2a    61 ............................................................
Rabbit_2b    61 ............................................................

Mouse_2a    121 EYEPEMGKVYRQDRKSVQRIKAKDIVPGDIVEIAVGDKVPADIRLTSIKSTTLRVDQSIL
Mouse_2b    121 ............................................................
Rat_2b      121 ............................................................
Rat_2a      121 ............................................................
Dog_2a      121 ............................................................
Cat_2a      121 ............................................................
Pig_2a      121 ............................................................
Pig_2b      121 ............................................................
Human_2b    121 ............................................................
Human_2c    121 ............................................................
Human_2a    121 ............................................................
Rabbit_2a   121 ............................................................
Rabbit_2b   121 ............................................................

Mouse_2a    181 TGESVSVIKHTDPVPDPRAVNQDKKNMLFSGTNIAAGKAMGVVVATGVNTEIGKIRDEMV
Mouse_2b    181 ............................................................
Rat_2b      181 ............................................................
Rat_2a      181 ............................................................
Dog_2a      181 ............................................................
Cat_2a      181 ............................................................
Pig_2a      181 ............................................................
Pig_2b      181 ............................................................
Human_2b    181 ............................................................
```

```
Human_2c    181 ..........................................................
Human_2a    181 ..........................................................
Rabbit_2a   181 ..........................................................
Rabbit_2b   181 ..........................................................

Mouse_2a    241 ATEQERTPLQQKLDEFGEQLSKVISLICIAVWIINIGHFNDPVHGGSWIRGAIYYFKIAV
Mouse_2b    241 ..........................................................
Rat_2b      241 ..........................................................
Rat_2a      241 ..........................................................
Dog_2a      241 ..........................................................
Cat_2a      241 ..........................................................
Pig_2a      241 ..........................................................
Pig_2b      241 ..........................................................
Human_2b    241 ..........................................................
Human_2c    241 ..........................................................
Human_2a    241 ..........................................................
Rabbit_2a   241 ..........................................................
Rabbit_2b   241 ..........................................................

Mouse_2a    301 ALAVAAIPEGLPAVITTCLALGTRRMAKKNAIVRSLPSVETLGCTSVICSDKTGTLTTNQ
Mouse_2b    301 ..........................................................
Rat_2b      301 ..........................................................
Rat_2a      301 ..........................................................
Dog_2a      301 ..........................................................
Cat_2a      301 ..........................................................
Pig_2a      301 ..........................................................
Pig_2b      301 ..........................................................
Human_2b    301 ..........................................................
Human_2c    301 ..........................................................
Human_2a    301 ..........................................................
Rabbit_2a   301 ..........................................................
Rabbit_2b   301 ..........................................................

Mouse_2a    361 MSVCRMFILDKVEGDTCSLNEFSITGSTYAPIGEVQKDDKPVKCHQYDGLVELATICALC
Mouse_2b    361 ..........................................................
Rat_2b      361 ......................T...................................
Rat_2a      361 ......................T...................................
Dog_2a      361 ..........R....S......T..........H........................
Cat_2a      361 ......................T...........H........................
Pig_2a      361 ......................T...........H........................
Pig_2b      361 ......................T...........H........................
Human_2b    361 ..........R...........T...........H......N................
Human_2c    361 ..........R...........T...........H......N................
Human_2a    361 ..........R...........T...........H......N................
Rabbit_2a   361 ............D.........T...........H........................
Rabbit_2b   361 ............D.........T...........H........................

Mouse_2a    421 NDSALDYNEAKGVYEKVGEATETALTCLVEKMNVFDTELKGLSKIERANACNSVIKQLMK
Mouse_2b    421 ..........................................................
Rat_2b      421 ..........................................................
Rat_2a      421 ..........................................................
Dog_2a      421 ..........................................................
Cat_2a      421 ..............K.F.........................................
Pig_2a      421 ..........................................................
Pig_2b      421 ..........................................................
Human_2b    421 ..........................................................
```

```
Human_2c    421 ..........................................................
Human_2a    421 ..........................................................
Rabbit_2a   421 ..........................................................
Rabbit_2b   421 ..........................................................
```

```
Mouse_2a    481 KEFTLEFSRDRKSMSVYCTPNKPSRTSMSKMFVKGAPEGVIDRCTHIRVGSTKVPMTPGV
Mouse_2b    481 ....................................................................
Rat_2b      481 ....................................................................
Rat_2a      481 ....................................................................
Dog_2a      481 ....................................................................
Cat_2a      481 ....................................................................
Pig_2a      481 ....................................................................
Pig_2b      481 ....................................................................
Human_2b   481 ....................................................................S..
Human_2c   481 ....................................................................S..
Human_2a   481 ....................................................................S..
Rabbit_2a  481 ....................................................................A..
Rabbit_2b  481 ....................................................................A..

Mouse_2a    541 KQKIMSVIREWGSGSDTLRCLALATHDNPLKREEMHLEDSANFIKYETNLTFVGCVGMLD
Mouse_2b    541 ....................................................................
Rat_2b      541 ............................R.......................................
Rat_2a      541 ............................R.......................................
Dog_2a      541 ...V........................R....N......................................
Cat_2a      541 ...V........................R....N......................................
Pig_2a      541 ............................MR....N.....................................
Pig_2b      541 ............................MR....N.....................................
Human_2b   541 ............................R...........................................
Human_2c   541 ............................R...........................................
Human_2a   541 ............................R...........................................
Rabbit_2a  541 ............................R......K.....................................
Rabbit_2b  541 ............................R......K.....................................

Mouse_2a    601 PPRIEVASSVKLCRQAGIRVIMITGDNKGTAVAICRRIGIFGQDEDVTSKAFTGREFDEL
Mouse_2b    601 ....................................................................
Rat_2b      601 ....................................................................
Rat_2a      601 ....................................................................
Dog_2a      601 ....................................................................
Cat_2a      601 ....................................................................
Pig_2a      601 ....................................................................
Pig_2b      601 ....................................................................
Human_2b   601 ....................................................................
Human_2c   601 ....................................................................
Human_2a   601 ....................................................................
Rabbit_2a  601 ..........................................................E....A.....
Rabbit_2b  601 ..........................................................E....A.....

Mouse_2a    661 SPSAQRDACLNARCFARVEPSHKSKIVEFLQSFDEITAMTGDGVNDAPALKKSEIGIAMG
Mouse_2b    661 ....................................................................
Rat_2b      661 ....................................................................
Rat_2a      661 ....................................................................
Dog_2a      661 ....................................................................
Cat_2a      661 ....................................................................
Pig_2a      661 N.....E.............................................................
Pig_2b      661 N.....E.............................................................
Human_2b   661 N.....................................................................A.......
Human_2c   661 N.....................................................................A.......
Human_2a   661 N.....................................................................A.......
Rabbit_2a  661 N.....................................................................A.......
Rabbit_2b  661 N.....................................................................A.......
```

```
Mouse_2a    721 SGTAVAKTASEMVLADDNFSTIVAAVEEGRAIYNNMKQFIRYLISSNVGEVVCIFLTAAL
Mouse_2b    721 ..........................................................
Rat_2b      721 ..........................................................
Rat_2a      721 ..........................................................
Dog_2a      721 ..........................................................
Cat_2a      721 ..........................................................
Pig_2a      721 ..........................................................
Pig_2b      721 ..........................................................
Human_2b    721 ..........................................................
Human_2c    721 ..........................................................
Human_2a    721 ..........................................................
Rabbit_2a   721 ..........................................................
Rabbit_2b   721 ..........................................................

Mouse_2a    781 GFPEALIPVQLLWVNLVTDGLPATALGFNPPDLDIMNKPPRNPKEPLISGWLFFRYLAIG
Mouse_2b    781 ..........................................................
Rat_2b      781 ..........................................................
Rat_2a      781 ..........................................................
Dog_2a      781 ..........................................................
Cat_2a      781 ..........................................................
Pig_2a      781 ..........................................................
Pig_2b      781 ..........................................................
Human_2b    781 ..........................................................
Human_2c    781 ..........................................................
Human_2a    781 ..........................................................
Rabbit_2a   781 ..........................................................
Rabbit_2b   781 ..........................................................

Mouse_2a    841 CYVGAATVGAAAWWFIAADGGPRVSFYQLSHFLQCKEDNPDFDGVDCAIFESPYPMTMAL
Mouse_2b    841 ..........................................................
Rat_2b      841 ..........................................E...............
Rat_2a      841 ..........................................E...............
Dog_2a      841 ......................................D.....E...............
Cat_2a      841 ......................................D.....E...............
Pig_2a      841 ......................T...............E.....V...........
Pig_2b      841 ......................T...............E.....V...........
Human_2b    841 ..........................................E...............
Human_2c    841 ..........................................E...............
Human_2a    841 ..........................................E...............
Rabbit_2a   841 ..........................................E...............
Rabbit_2b   841 ..........................................E...............

Mouse_2a    901 SVLVTIEMCNALNSLSENQSLLRMPPWENIWLVGSICLSMSLHFLILYVEPLPLIFQITP
Mouse_2b    901 ..........................................................
Rat_2b      901 ..........................................................
Rat_2a      901 ..........................................................
Dog_2a      901 ..........................................................
Cat_2a      901 ..........................................................
Pig_2a      901 ..........................................................
Pig_2b      901 ..........................................................
Human_2b    901 ..........................................................
Human_2c    901 ..........................................................
Human_2a    901 ..........................................................
Rabbit_2a   901 ..........................................................
Rabbit_2b   901 ..........................................................
```

```
Mouse_2a    961 LNLTQWLMVLKISLPVILMDETLKFVARNYLEQPAILE----------------------
Mouse_2b    961 ..............................GKECVQPATKSSCSLSACTDGISWPF
Rat_2b      961 ..............................-.GKECA.....P.............
Rat_2a      961 ..............................-.AILE
Dog_2a      961 ..............................-.AILE
Cat_2a      961 ..............................-.AILE
Pig_2a      961 ..............................-.AILE
Pig_2b      961 ..............................-.GKEC.......-..F..........
Human_2b    961 ..V...........................-.GKEC.......-..F..........
Human_2c    961 ..V...........................-.VLSSL
Human_2a    961 ..V...........................-.AILE
Rabbit_2a   961 ..V...........................-.AILE
Rabbit_2b   961 ..V...........................-.GKEC....PQ.-...W...E.V....

Mouse_2b   1021 VLLIMPLVVWVYSTDTNFSDMFWS
Rat_2b     1020 ........................
Pig_2b     1019 ........................
Human_2b   1019 ........................
Rabbit_2b  1019 ....V...M...........LL..
```

Fig. 3 Targeting construct for Serca2 flox gene modification. Sequence information.

---------- Serca2 gene ----------

LoxP site 1: Intron 1
underlined sequence = loxP site and cloning sequence

Exon I (partial)

CACGGGGCTGAGCTTGGAGCAGGTCAAGAAGCTCAAGGAGAGATGGGGCTCCAACG*gtaggtgcggggcgccggggct*
gcagcggcgcggcgcgggcccgagcgccaaggaagatggctgacccggctccacctcgtggggcttggctcggcgcgg
cgcccgacggctgcgagaggccggcggtccacgcgcgggtctgggccatcgccgaccttaggggtctcgaatcaagct
tatcgataccgtcgatcggacctcgaggggggggcccggtacccggggatcaattcgagctcgcccggggatcgatccg
gaacccttaat**ATAACTTCGTATAATGTATGCTATACGAAGTTAT**taggtccctcgacctgcagcccaagctccGGGG
Atctcgagccggtgaccttcccggccggcgctcagcgagtcggattgggggggggggagagggagtgggaaggaggga
ggaggttcctgcggctgggctgagtccccgcggatttatgaggcgtcgatgttgttgagaaaccctcggaccgtttc
ttgtgctccccaaagttgcacatctggcagaagtgatgacccagctgaaatgactgcatggtcctggaggccggagag
ggcttacgggcagttccgaggccactgattaccagggctgaataattttctcggggtatcaaagtggagacagattgt
tgtacgttcatacacctatatccgccattcagacaacgatggtggtgaatttagcagttttaataaaagcgctaata
caatatcttcatttttctttc

---------- Serca2 gene ----------

LoxP site 2: Intron 3 5′of genomic XcmI site
underlined sequence = loxPsite, cloning sites and partial HSV-TK

ccaatttttattcttagaacattgtattcttatactgtgataggaagtgaaaaatcatacagtacttgtcttaggttt
cacaaaaactgataactgtatggtttcaattatgtattcacacgtttaagtctgacccagggGGATCCggaacccttaa
t**ATAACTTCGTATAATGTATGCTATACGAAGTTAT**taggtccctcgacctgcagcccaagctgatcctctagtcgagc
cccagctggttctttccgcctcagaagccatagagcccaccgcatccccagcatgcctgctattgtcttcccaatcct
ccccttgctgtcctgccccaccccacccccagaatagaatgacacctactcagacaatgcgatgcaatttcctcat
tttattaggaaaggacagtgggagtggcaccttccagggtcaaggaaggcacgggggagggcaaacaacagatggct
ggcaactagaaggcacagtcgaggctgatcagcgagctctagctagagaattgatccccctcagaagaactcgtcaaga
aggcgatagaaggcgatgcgctgcgaatcgggagcggcgat*ccgtaaagcacgaggaagcgg*cagcccattcgccg
ccaagctctttcagcaatatcacgggtagccaacgctatgt*ctgataagcggtccgcccacacccaa*ccggccaca
agtc*atgaaatcca*aaaaagcgggccatttttccacc*atgatttt*cggcaagcaaggcctttt*ccattgggtca
ccgac*aga*catt*tccgt*c*ggcattgcgc*ccct

---------- HSV-TK Neo antibiotics cassette ----------

LoxP site 3: Intron 3 5′of genomic XcmI site
underlined sequence = loxP site, cloning sites and partial Neo gene

gttttcat*accaccgcgggtcccgggc*gatat*ttcaccttgtc*ag*cggtgttgtgtggtgtaaatgttcgcgat
tgtttcgaaagcccc*agcacccgccagtaagtcatcggctcgggtacgtagacgatatcgtcgcgcgaacccagggcc
accagcaagttgcgtggtggtggttttccccatcc*gtggggac*gtctatataaaacc*gcagtagcgtgggcattttc
tgctccgggcggacttccgtggcttcttgctgccggcgagggcgcaacgccgtacgtcggttgctatggccgcgagaac
gcgcagcctggtcgaacgcagacgcgtgttgatggccggggtacgaagccatacgcgcttctacaaggcgctggccgaa
gaggtgcgggagtttcacgccaccaagatctgcggcacgctgttgacgctgttaagcgggtcgctgcagggtcgctcgg
tgttcgaggccacacgcgtcaccttaatatgcgaagtggacctcggaccgcgccgccccgactgcatctgcgtgttcga
attcgccaatgacaagacgctgggcggggtttgctcgacattgggtggaaacattccaggcctgggtggagaggctttt
tgcttcctcttgcaaaaccacactgctcgacattgggtggaaacattccaggcctgggtggagaggcttttgcttcct
cttgaaaaccacactgctcgatccggaacccttaat**ATAACTTCGTATAATGTATGCTATACGAAGTTAT**tag
gtccctcgacctgcagcccaagctgatcctctagagtcgacctcga*tctgtggtcatggcctctatgaaaacattagcttagagg*

Fig. 4A **Schematic representation of genetic manipulation.**

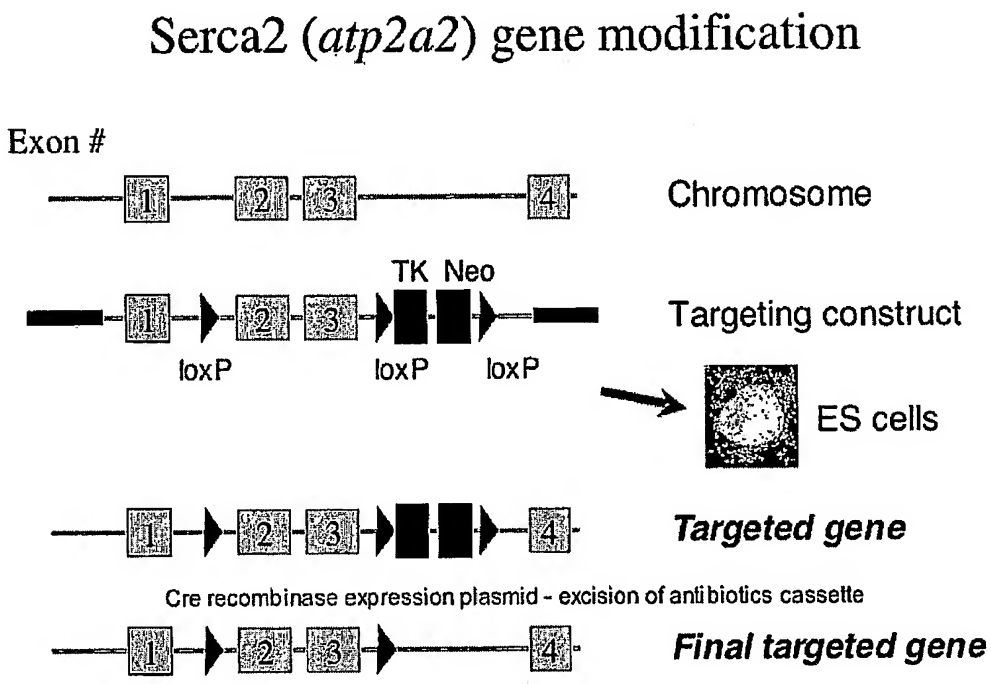


Fig 4 B: Verification of Serca locus targeting events offspring from chimeric mice.

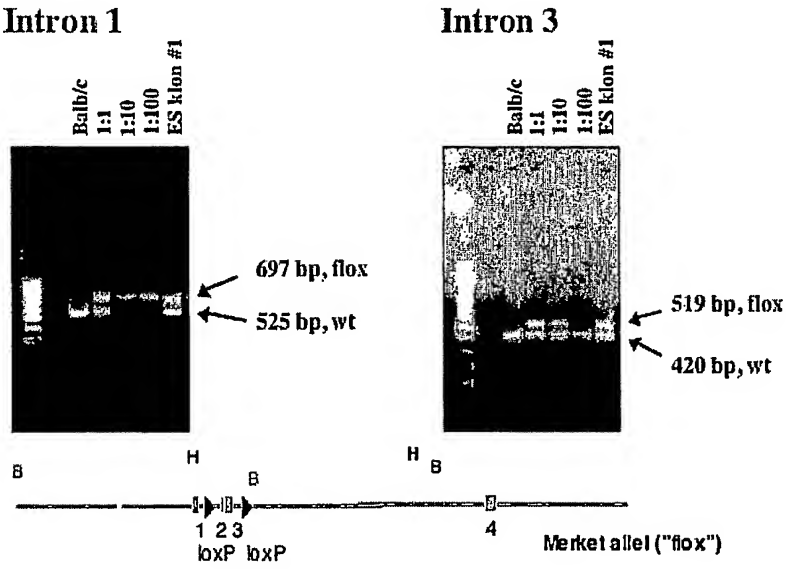
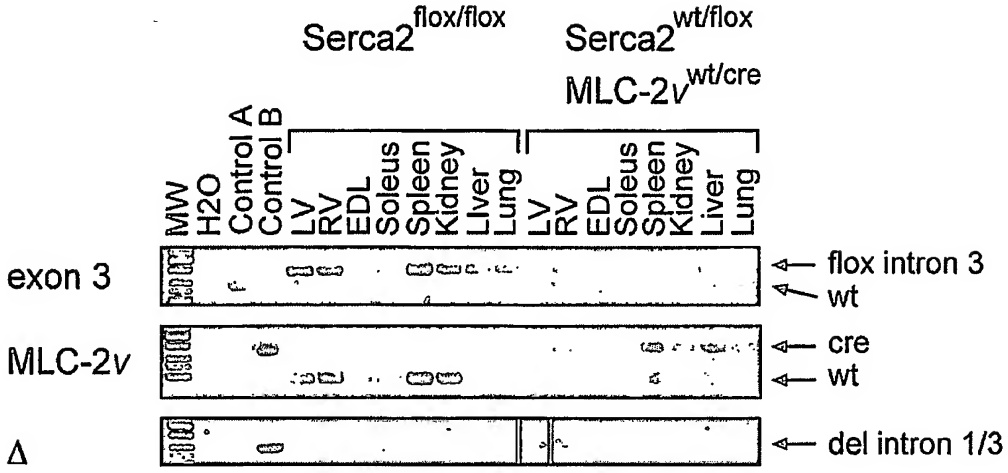


Fig. 5 Specificity of gene deletion in a test model.



Control A = wt ES cells (14.1a)

Control B = Upper panel: Serca2$^{wt/flox}$ ES cells

Middle panel: Left ventricle from MLC-2V-Cre mice

Lower panel: Serca2$^{wt/del}$ ES cells

LV = heart left ventricle

RV = heart right ventricle

EDL = extensor digitorum longus muscle (fast-twitch skeletal muscle)

Soleus = soleus muscle (slow-twitch skeletal muscle)

Other tissues as indicated.

Fig. 6 Cardiac ANP mRNA expression.

Fig. 7 Serca2 protein expression.

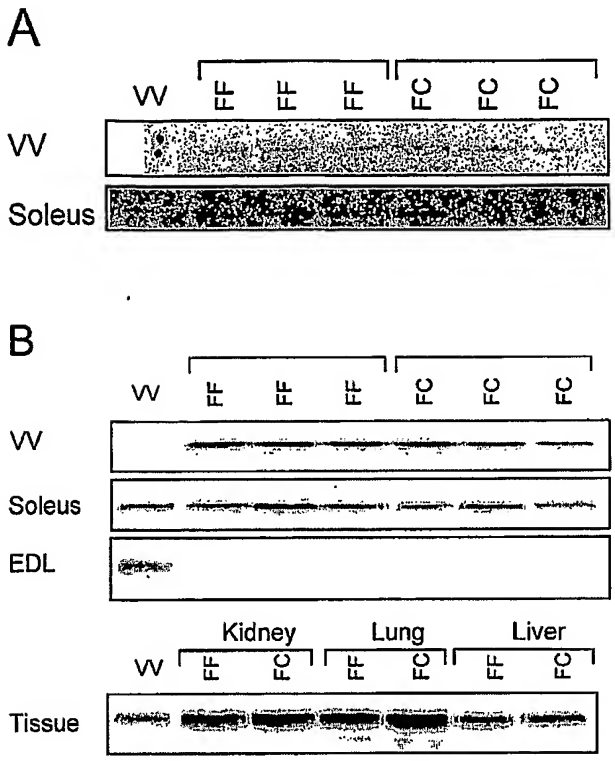


Fig. 8 Compensatory mechanisms in Serca^{flox} MLC-2v-Cre mice.